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Yeast chitin synthase 2 activity is modulated by proteolysis and phosphorylation

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Saccharomyces cerevisiae Chs2 (chitin synthase 2) synthesizes the primary septum after mitosis is completed. It is essential for proper cell separation and is expected to be highly regulated. We have expressed Chs2 and a mutant lacking the N-terminal region in *Pichia pastoris* in an active form at high levels. Both constructs show a pH and cation dependence similar to the wild-type enzyme, as well as increased activity after trypsin treatment. Using further biochemical analysis, we have identified two mechanisms of chitin synthase regulation. First, it is hyperactivated by a soluble yeast protease. This protease is expressed during exponential growth phase, when budding cells require Chs2 activity. Secondly, LC-MS/MS (liquid chromatography tandem MS) experiments on purified Chs2 identify 12 phosphorylation sites, all in the N-terminal domain. Four of them show the perfect sequence motif for phosphorylation by the cyclin-dependent kinase Cdk1. As we also show that phosphorylation of the N-terminal domain is important for Chs2 stability, these sites might play an important role in the cell cycle-dependent degradation of the enzyme, and thus in cell division.

Key words: chitin synthase, overexpression, regulation, phosphorylation, post-translational modification, proteolytic activation.

INTRODUCTION

Chitin, a β-1,4-linked polymer of *N*-acetylglucosamine, is a polysaccharide that has an important structural role in many organisms, such as arthropods, molluscs, cephalopods, and fungi. The amounts of chitin present vary between these organisms; in yeast it constitutes only 1% of the cell wall, but still is an essential component [1,2]. Chitin is synthesized from UDP-GlcNAc (UDP-*N*-acetylglucosamine) by chitin synthases (EC 2.4.1.16). Typically, these enzymes consist of three domains [3]: a hydrophilic N-terminal domain, a highly conserved central catalytic domain and a hydrophobic carboxy-terminal domain integrated into the membrane (Figure 1). Chitin synthases are classified as part of the glycosyl transferase family 2 [4,5], a sequence family that contains a wide range of enzymes. Besides chitin synthases, the most notable examples are the synthases of the structural polysaccharides cellulose, hyaluronan, β-1,3-glucan and β-1,4-mannan. The catalytic domains of the glycosyl transferase family 2 share a GT-A fold [6].

Saccharomyces cerevisiae contains three membrane-bound chitin synthases. Each enzyme has its own specific function: Chs1 has a repair function during cell separation [7], Chs2 is essential for primary septum formation and thus for cell division [8], and Chs3 synthesizes the chitin ring at bud emergence, as well as the chitin in the cell wall [2]. Since each chitin synthase acts at a different stage of the cell cycle, independent mechanisms for their regulation must exist in the cell. Disruption of specific chitin synthase genes also results in different effects. *S. cerevisiae* cells lacking Chs1 grow normally, without showing apparent loss of cell wall chitin content [9]. Likewise, disruption of the Chs3 gene was not lethal [10]. Therefore, these enzymes do not seem to be critical for yeast cell survival. On the other hand, disruption of Chs2 causes severe growth defects and morphological abnormalities [11,12]. The regulation of Chs2 is different from that of the other two chitin synthases: its concentration in the cell changes greatly during the cell cycle, in contrast with the constant levels of Chs1 and Chs3 [13–15]. So, whereas regulation of Chs1 and Chs3 can only be post-translational, Chs2 is also significantly regulated by synthesis and degradation.

Chs2 is especially interesting, as it shows 55% sequence identity and is functionally analogous to chitin synthase 1 from the human pathogen *Candida albicans*, which is essential for cell survival [12]. The importance of these enzymes for their organisms, together with the lack of chitin synthases in humans, makes them excellent targets for antifungal drugs.

In this paper we report the heterologous expression and characterization of Chs2. As it had been previously shown that the N-terminal 222 amino acid residues of Chs2 could be truncated without loss of activity or function [16], we have also over-expressed the active mutant Chs2ΔN222. Biochemical characterization of both proteins allows further insights in Chs2 regulation by post-translational modification, as well as the role of the N-terminal domain.

EXPERIMENTAL PROCEDURES

Bacterial and yeast constructs

Genomic DNA from *S. cerevisiae* strain Y3437 was used for PCR amplification of the Chs2 and Chs2ΔN222 genes. The primers used for amplifying Chs2 were 5′-ATTGCACATATGACGAGA-AACCCGTTTATGGTGG-3′ and 5′-TCCTCGAGGCCCTTTTT-GTGGAAAACATTTG-3′, containing NdeI and XhoI sites respectively (underlined). For amplification of Chs2ΔN222, the primers were 5′-ATTGCACATATGGTCTCAGACTTGCCTCC-C-3′ and 5′-TCCTCGAGGCCCTTTT-TGTGGAAAACATTTG-3′, containing NdeI and XhoI sites respectively (underlined). For bacterial expression, the two constructs were inserted into the

Abbreviations used: Cdk, cyclin-dependent kinase; Chs, chitin synthase; GlcNAc, *N*-acetylglucosamine; IMAC, immobilized metal-ion-affinity chromatography; LC-MS/MS, liquid chromatography tandem MS.

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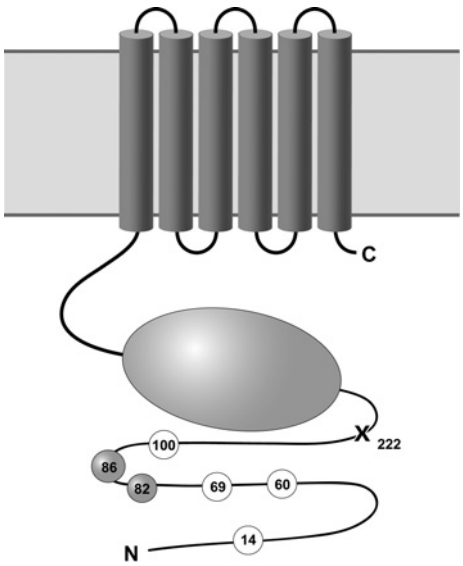

Figure 1 Schematic drawing of the domain organization of Chs2

The N-terminal soluble domain and the central catalytic domain are located in the cytoplasm; the hydrophobic C-terminal domain is integrated into the plasma membrane. Shaded circles indicate the positions of observed phosphorylation sites [40], clear circles show those of the consensus phosphorylation sequences for Cdk1. Residue 222, the end of the region that is deleted in the Chs2ΔN222 mutant, is indicated by a cross.

NdeI and XhoI sites of the pET26b vector (Novagen). For expression in *Pichia pastoris*, the constructs were cloned in a modified pPICZ B vector (Invitrogen) containing a Strep 2 tag, prescission cleavage site, multiple cloning site for NdeI and XhoI restriction sites, prescission cleavage site, and His_{10} tag; for this system expression is under the control of the alcohol oxidase (AOX1) promoter. All constructs contain a poly-histidine tag for protein detection by Western blot analysis. The correct insertion and sequence of each construct was confirmed by DNA sequencing.

P. pastoris culture conditions

The protease-deficient *P. pastoris* strain SMD1163 (Invitrogen) was used for expression of the recombinant genes. The higher expressing clones were selected according to [17] and made into His^+ mutants by transformation with a pPIC3.5 vector. For expression, SMD1163 cells carrying the foreign gene were grown overnight in MGY (minimal glycerol) medium [0.34 % (w/v) yeast nitrogen base, 1 % (w/v) ammonium sulphate, 1 % (w/v) glycerol and 4×10^{-5} % (w/v) biotin] to a D_{600} of 2–6. The cells were centrifuged at 1500 ***g*** for 10 min, then suspended to a D_{600} of 1–2 in MM (minimal methanol) medium [0.34 % (w/v) yeast nitrogen base, 1 % (w/v) ammonium sulphate, 1.64 μM biotin and 0.5 % (v/v) methanol] and grown for 24 h at 30 °C. Cells were harvested by centrifugation at 1500 ***g*** for 10 min and the pellets stored at −80 °C or used immediately for further studies.

P. pastoris membrane preparation

Cells were suspended to a D_{600} of 50–100 in 30 mM Hepes, pH 7.5, and 100 mM NaCl. After addition of an equal volume of ice-cold acid-washed glass beads (0.5 mm diameter), the cells were broken by vortex mixing for eight 30 s bursts separated by 30 s cooling on ice. Glass beads, cell debris and unbroken cells were removed by centrifugation at 1500 ***g*** for 10 min and the pellet was washed with an equal volume of buffer and centrifuged again. To separate the membrane fraction from the soluble components, the combined supernatants were then centrifuged at 100 000 ***g*** for 30 min. The crude membrane pellet was suspended in 30 mM Tris or Hepes buffer, pH 7.0, 7.5 or 8.0, and the protein content determined using the DC protein assay (BioRad).

Bacterial culture conditions

Chs2 and Chs2ΔN222 were expressed in *Escherichia coli* C41 (Avidis). Bacterial cells were grown at 37 °C in TB (Terrific broth) medium until the D_{600} had reached 0.6–0.8, and then induced with 2 mM IPTG (isopropyl β-D-thiogalactoside) for 3–5 h. Cells were harvested by centrifugation at 3300 ***g*** for 15 min and used immediately for membrane preparation.

Bacterial membrane preparation

Cells were suspended in 30 mM Tris/HCl (pH 7.5) and 150 mM NaCl. The cells were then passed three times through a Microfluidiser model M-110L (Microfluidics, Newton, MA, U.S.A.) equipped with a 110 μm interaction chamber and a cooling coil, which were immersed in water at 0 °C. After centrifugation at 15 000 ***g*** for 10 min, the supernatant was centrifuged at 100 000 ***g*** for 1 h. The pellet was suspended in 30 mM Tris/HCl (pH 7.5), and the protein content was determined as above.

S. cerevisiae culture conditions

S. cerevisiae wild-type strain YPH419 were grown overnight in YPD medium [1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) dextrose] to $D_{600} = 0.5$ (exponential phase) or to $D_{600} = 14$ (stationary phase). The *P. pastoris* membrane preparation protocol was followed, and the supernatant of the 100 000 ***g*** centrifugation step (soluble fraction) was stored at −80 °C or used immediately.

Activity assay

The assay for measuring chitin synthase activity was carried out according to a previously described method [18,19] in a 50 μl reaction mixture. Unless otherwise indicated, it contained 30 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$ (for Chs2ΔN222) or 5 mM $MnCl_2$ (for Chs2), 32 mM GlcNAc, 1 mM UDP-[U-^{14}C]-GlcNAc (Amersham, specific radioactivity 553 000 d.p.m./μmol), and 25 μg of *P. pastoris* or 800 μg of *E. coli* membranes. For investigation of the activity at a pH range of 6–9, a mixture of buffers was used. This mixture contained 30 mM Hepes, 30 mM Mes and 30mM Taps. To test bivalent cation specificity, 5 mM of a cation salt ($MgCl_2$, $MnCl_2$, $CoCl_2$, $NiCl_2$ or $CdCl_2$) or 5 mM EDTA was used in the reaction mixture. Samples where incubated at 30 °C for 30 min for *P. pastoris*, or 16–20 h for *E. coli* membranes. To measure background activity from the chitin synthases of *P. pastoris,* membranes that did not contain overexpressed chitin synthases were used. Mean values and errors (standard deviation) were calculated from two sets of three independent experiments, using the program OriginPro 7.5.

Chitin synthesis was stopped by the addition of 1 ml of ice-cold 10 % trichloroacetic acid, and the insoluble chitin was collected by filtration through a GF/B glass-fibre disc (Whatman) previously soaked in 10 % trichloroacetic acid. The filters were subsequently washed three times with 1 ml of ice-cold 10 % trichloroacetic acid, and twice with 1 ml of ice-cold 66 % ethanol, according to [20]. Filters were then transferred to scintillation fluid

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(Rotiscint eco plus, Roth). Synthesized chitin was quantified by determining the radiation level of the filter using the scintillation counter TRI-CARB 1500 (Canberra-Packard).

Proteolytic activation

For activation of Chs2 and Chs2ΔN222 by trypsinization, *P. pastoris* membranes were incubated with trypsin (Sigma–Aldrich) for 10 min at 30 °C. As previously reported [18,21], the optimal amount of trypsin needed to be ascertained for each batch of membranes. The optimal trypsin/total membranes ratio varied from 1:30 to 1:150. After the 10 min incubation, proteolysis was stopped by adding a 2-fold excess of soybean trypsin inhibitor (Fluka).

To activate Chs2 and Chs2ΔN222 using the soluble *S. cerevisiae* fraction, the soluble fraction was incubated with trypsin for 10 min at 30 °C, after which a 2-fold excess of trypsin inhibitor was added. This mixture was then added to the chitin synthase-expressing membranes and incubated for 10 min at 30 °C.

Inactivation of soluble *S. cerevisiae* fraction

The trypsin-activated soluble *S. cerevisiae* fraction was incubated with each of the following protease inhibitors: Pefabloc® (100 μM), E-64 (15 μM), leupeptin (400 μM), benzamidine (100 μM) or pepstatin A (20 μM). After this, the mixture was added to Chs2-containing membranes and the activity assay was carried out.

Protein dephosphorylation

P. pastoris membranes (100 μg) were incubated with 2 units of calf intestine alkaline phosphatase (Roche) for 16 h at 4 °C.

Partial protein purification

P. pastoris membranes containing overexpressed Chs2 were diluted in solubilization buffer [200 mM NaCl, 30 mM imidazole pH 7.5, 10 % (w/v) glycerol and 1 % Fos-choline-14] plus protease inhibitors (0.1 mg/ml trypsin inhibitor, 1 mM benzamidine and 0.1 mM Pefabloc®) and incubated for 1 h at 4 °C. The unsolubilized material was removed by centrifugation for 1 h at 170 000 ***g*** and loaded on to a Ni-Sepharose (Amersham) column. The column was washed with wash buffer (70 mM imidazole, pH 7.5, 150 mM NaCl and 0.05 % Fos-choline-14) and eluted with buffer containing 300 mM imidazole, pH 7.5, 150 mM NaCl and 0.05 % Fos-choline-14.

SDS/PAGE

Resolving gels (10 %) were prepared and overlaid with a 3 % stacking gel. Before electrophoresis, the proteins were denatured in sample buffer (60 mM Tris/HCl, pH 6.8, 1.5 % SDS, 10 % glycerol, 0.005 % Bromophenol Blue and 50 mM dithiothreitol).

Western blotting

After SDS/PAGE, proteins were transferred on to PVDF membrane (Immobilon™ pore size 0.45 μm, Millipore) using a semi-dry transfer apparatus (Trans-Blot®SD, Bio-Rad). For immuno-detection, proteins were probed with monoclonal anti-poly-histidine antibody (Sigma–Aldrich) and visualized with anti-mouse alkaline phosphatase-conjugated secondary antibody (Sigma–Aldrich), both antibodies were diluted 1:1000 in 20 mM Tris/HCl, pH 7.5, plus 150 mM NaCl.

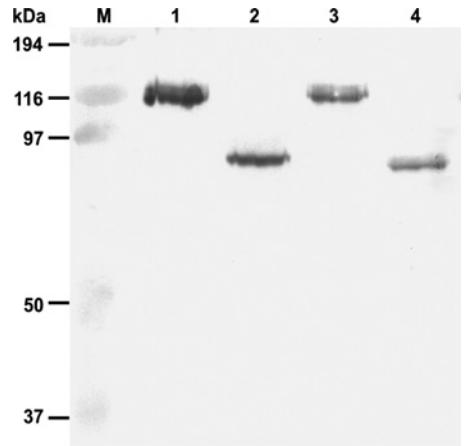

Figure 2 Western blot analysis of membranes shows that, in both expression systems, the recombinant proteins are integrated into the membrane

Each protein migrates according to its sequence-based predicted molecular mass, and to the same level when expressed in yeast or bacteria, which implies that both are not glycosylated in *P. pastoris*. The His-tagged proteins were probed with monoclonal anti-poly-histidine antibody and visualized with alkaline phosphatase-conjugated secondary antibody. Lane M, molecular-mass marker in kDa; lane 1, Chs2 (115 kDa) in *P. pastoris*; lane 2, Chs2ΔN222 (90 kDa) in *P. pastoris*; lane 3, Chs2 in *E. coli*; lane 4, Chs2ΔN222 in *E. coli*.

Phosphorylation site mapping

The partially purified protein was resolved by SDS/PAGE analysis and the gel fraction containing Chs2 was cut out for phosphopeptide mapping. This analysis was done at the Center for Functional Genomics (University at Albany, Rensselaer, NY, U.S.A.). For this purpose, the protein was in-gel tryptic digested and the resulting phosphopeptides were enriched by TiO_2 IMAC (immobilized metal-ion-affinity chromatography). LC-MS/MS (liquid chromatography tandem MS) was performed on the enriched fraction to identify phosphorylation sites, and on the entire sample to determine the sequence coverage.

Protein phosphorylation

P. pastoris membranes (150 mg) were incubated with 20 units of M-phase Cdc2-cyclin B (NEB, recognition motif S/T-P-x-K/R) for 30 min at 30 °C in a 30 μl reaction mixture containing 200 μM [γ-^{32}P]ATP (Perkin-Elmer, 3 mCi/μmol) and protease inhibitors (0.1 mg/ml trypsin inhibitor, 1 mM benzamidine and 0.1 mM Pefabloc®). After SDS/PAGE, phosphorylated protein was detected by autoradiography.

RESULTS AND DISCUSSION

We established the heterologous expression of the *S. cerevisiae* Chs2 and the deletion mutant Chs2ΔN222 in *P. pastoris* and *E. coli*. To study the proteins further, we isolated membranes from both expression systems. As observed by immunodetection (Figure 2), the proteins migrate on SDS/PAGE with the molecular masses expected from their amino acid sequences (115 and 90 kDa for Chs2 and Chs2ΔN222, respectively, including affinity tags), showing that neither Chs2 nor Chs2N222 is glycosylated. This is consistent with a Chs2ΔN193 mutant expressed in *S. cerevisiae* that was shown not to be glycosylated [22], and is

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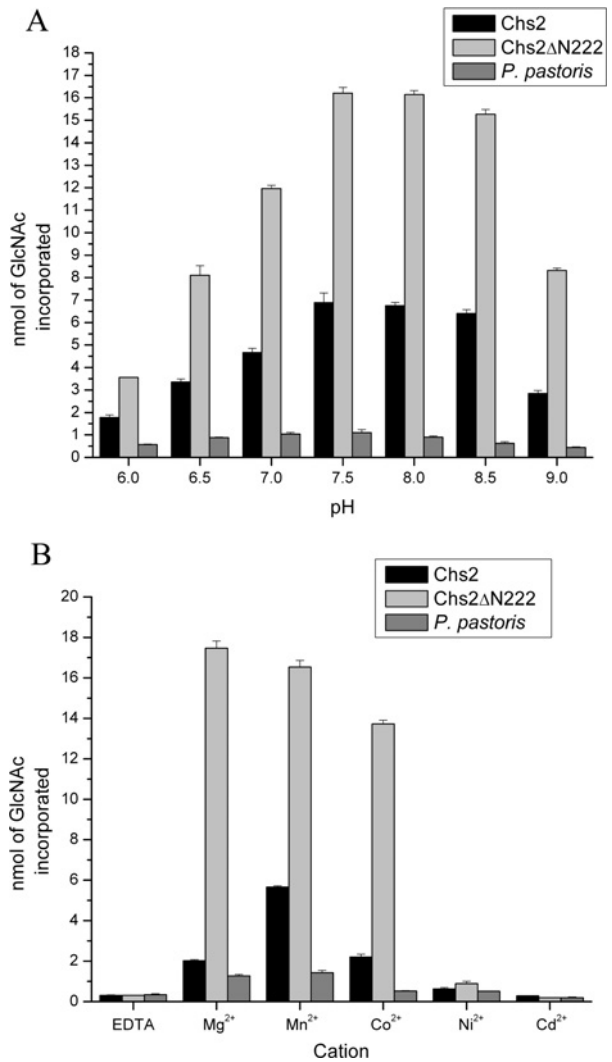

Figure 3 Biochemical characterization of *P. pastoris*-expressed Chs2 and Chs2ΔN222 shows identical enzymatic properties as compared with the wild-type protein

Black and light-grey bars show the activities of Chs2 and Chs2ΔN222 respectively. Dark-grey bars show the background activity of native chitin synthases of *P. pastoris*. (**A**) The dependence of protein activity on pH, in the presence of 5 mM $MnCl_2$. To ascertain that all differences measured were due only to pH, for each experiment the same buffer mixture (30 mM Hepes, 30 mM Mes and 30 mM Taps) was used. (**B**) The effect of cations on protein activity, assayed at pH 7.5. Each bar comprises two independent sets of three experiments ± S.D. ($n = 6$).

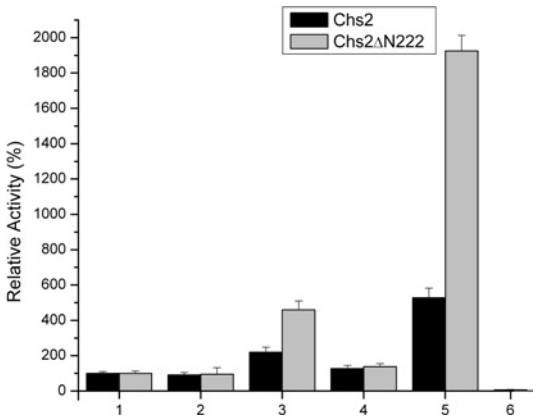

Figure 4 Hyperactivation of *P. pastoris*-expressed Chs2 (black) and Chs2ΔN222 (grey) by trypsin and/or activated soluble fraction of *S. cerevisiae*

Activities are shown relative to their corresponding: (1) basal activities (non-treated membranes, 6.0 and 5.5 nmol of GlcNAc incorporated for Chs2 and Chs2ΔN222, respectively, each set to 100 %); (2) activities of membranes in the presence of the soluble *S. cerevisiae* fraction; (3) activities of membranes in the presence of a trypsin-activated soluble *S. cerevisiae* fraction; (4) activities of membranes in the presence of inhibited trypsin; (5) activities of trypsin-treated membranes; and (6) shows the activity of trypsin-activated soluble *S. cerevisiae* fraction by itself. Each bar comprises two independent sets of three experiments ± S.D. ($n = 6$).

further confirmed by the observation that the proteins expressed in *E. coli*, which lacks the glycosylation machinery of yeast, have molecular masses comparable with the *P. pastoris*-expressed proteins (Figure 2).

Chs2 and Chs2ΔN222 expressed in both expression systems are active. However, the activities of *E. coli*-expressed proteins were significantly lower than those from *P. pastoris*. Longer incubation time and higher amounts of membranes were needed to measure similar levels of activity. Therefore, for further characterization, only the *P. pastoris*-expressed proteins were used. *P. pastoris* is a yeast with a chitinous cell wall similar to *S. cerevisiae*, and indeed, *P. pastoris* membranes not expressing Chs2 show chitin synthase activity. This activity is, however, negligible compared with that of overexpressed Chs2 or Chs2ΔN222 (Figure 3).

To determine whether both Chs2 and Chs2ΔN222 show enzymatic characteristics similar to the wild-type protein, their dependence on both pH and bivalent cations was investigated (Figure 3). The *P. pastoris*-expressed Chs2 and Chs2ΔN222 activities are strictly dependent on bivalent cations (Figure 3B). For Chs2, we obtained the highest activity in the presence of Mn^{2+}, and reduced activity with Mg^{2+} and Co^{2+}. Chs2ΔN222 showed high activity in the presence of Mn^{2+} and Mg^{2+}, with a slight preference for Mg^{2+}, and, similar to the full-length protein, less activity with Ni^{2+} and Co^{2+}. For all constructs, activity was lost upon addition of EDTA or Cd^{2+} to the reaction mixture.

This preference for Mn^{2+} or Mg^{2+} fits with X-ray structures of glycosyl transferases with the GT–A fold, which show that either of these two cations bind the two phosphate groups of the UDP-sugar substrate, and are thought to assist in cleaving the sugar–UDP bond [6,23]. The decrease of activity using the various bivalent cations matches the decrease of their ionic radii, and also corresponds to the cation selectivity of wild-type Chs2 and *S. cerevisiae*-expressed mutants [18,22].

Besides the cation dependence, our two constructs also share an optimum pH between 7.5 and 8.5 with the wild-type enzyme (Figure 3A) [11,18,22,24]. Together, these results show that in *P. pastoris*, the protein is expressed in a functional form, having the characteristics of the wild-type enzyme. Moreover, when treated with appropriate amounts of trypsin, both proteins show high levels of hyperactivation (Figure 4); this too is consistent with the wild-type enzyme [16,18]. Interestingly, the two proteins exhibit different levels of proteolytic hyperactivation: 18-fold for Chs2ΔN222 compared with 6-fold for Chs2 (Figure 4). The increased activation level for Chs2ΔN222 as compared with Chs2 implies that the N-terminal domain obstructs trypsin activity, and that its removal could lead to a more exposed activation site.

Regulation of Chs2

Chs2 synthesizes the primary septum separating mother and daughter cell after contraction of the actomyosin ring [25,26], at a very specific time and position. This requires strong regulation of Chs2 activity. Most obviously, the expression and degradation of the enzyme are under tight control, resulting in a half-life

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of only 25 min [14]. Although the expression of Chs2 peaks during mitosis [13], the protein accumulates in the endoplasmic reticulum until mitotic exit, when it is transported to the mother–bud neck [27]. It already disappears from the neck 8 min later [27,28], to be degraded by the major vacuolar protease PEP4 [14].

In addition to this metabolic regulation, post-translational modification has been observed for Chs2. Chs2 is phosphorylated *in vitro* by, and thus is a possible *in vivo* target of, the cyclin-dependent kinase Cdk1 in complex with the mitotic phase cyclin Clb2 [29,30]. Also, the *in vitro* chitin synthase activity is increased markedly by proteolysis [18].

Proteolytic activation

There is a long-standing question about the putative zymogenic form of chitin synthases. For *S. cerevisiae*, the three chitin synthases are active in their unprocessed form. Trypsin has been reported to stimulate chitin synthase activity of Chs1 and Chs2 [18,31], but not of Chs3 [24]. In the case of Chs1, the stimulation was 7- to 15-fold [18,31], and trypsin treatment of Chs2 shows approx. 4-fold increased activity [18]. In the case of the insect *Manduca sexta*, trypsin does not activate chitin synthesis directly. However, trypsin was found to activate a soluble stimulatory factor in the midgut, which increased chitin synthesis by 25–30 % [32]. For our *P. pastoris*-expressed constructs, we measured a 6-fold activation by trypsinization for the full-length protein, and 18-fold for the N-terminal deletion mutant (Figure 4, condition 5).

Even though hyperactivation by proteolysis has been well established for Chs1 and Chs2, no endogenous protease that could take this role has been identified. Chs1 was reported to be activated by *S. cerevisiae* proteinase B [31,33]. However, proteinase B did not influence Chs1 activity *in vivo* [34,35], demonstrating that the observed proteolytic stimulation is either not physiologically relevant, or performed by another protease.

We tested the possibility of hyperactivation of Chs2 by factors present in the soluble fraction of *S. cerevisiae* cells, using cells with exponential growth rate, harvested at a D_{600} of 0.5. We found that incubation with the soluble fraction of *S. cerevisiae* cells in itself does not influence Chs2 activity in *P. pastoris* membranes. However, when the soluble fraction is first treated with trypsin, and then, after inhibition of trypsin, is added to the *P. pastoris* membranes, it does increase Chs2 and Chs2ΔN222 activity 2- to 4-fold (Figure 4, condition 3).

A number of controls were necessary to confirm that there was indeed an activating factor in the soluble fraction. First, the trypsin-activated soluble fraction did not show any chitin synthase activity by itself (Figure 4, condition 6), excluding the possibility that the higher activity is caused by trypsin-activated chitin synthase in the soluble fraction. Second, no hyperactivation was observed when trypsin was inhibited before being added to the membranes (Figure 4, condition 4), showing that there was no residual trypsin activity.

Since trypsin activity in the trypsinized soluble fraction has been inhibited prior to adding this mixture to the membranes, the activating factor can only be a part of the soluble *S. cerevisiae* fraction. Moreover, the need for a protease to trigger the stimulatory effect implies that the activating factor in the soluble fraction is a protein. To test whether this soluble protein could be, similar to trypsin, a protease, we investigated the effect of a range of protease inhibitors on the activated soluble *S. cerevisiae* fraction. Before addition to the Chs2-containing membranes, the trypsin-activated soluble *S. cerevisiae* fraction was incubated with each of the following protease inhibitors: Pefabloc®, E-64, leupeptin, benzamidine or pepstatin A. Indeed,

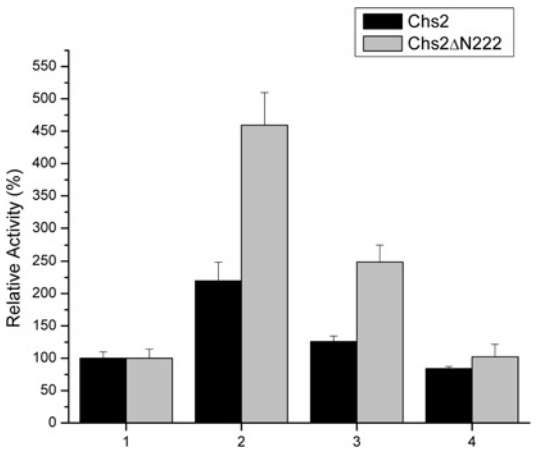

Figure 5 Activation of *P. pastoris*-expressed Chs2 and Chs2ΔN222 by the *S. cerevisiae* soluble fraction is inhibited by leupeptin

Activity of Chs2 (black) and Chs2ΔN222 (grey) is shown relative to: (1) basal activity (non-treated membranes, set to 100 %); (2) membranes treated with the activated soluble *S. cerevisiae* fraction; (3) activated soluble *S. cerevisiae* fraction inhibited by 400 μM leupeptin before addition to membranes; (4) membranes treated with 400 μM leupeptin. Each bar comprises two independent sets of three experiments ± S.D. ($n = 6$).

the addition of leupeptin, a serine and cysteine protease inhibitor, almost completely abolished the stimulatory effect, while it did not affect the activity of Chs2 or Chs2ΔN222 by itself (Figure 5).

Identification and isolation of this yeast protease and genetic experiments as performed in [35] will be necessary to understand the physiological role of this phenomenon. However, some characteristics of the activating protease can already be defined. First, the inhibition of activation by leupeptin shows that the enzyme in question is a serine or cysteine protease. Second, activation was only observed when the soluble *S. cerevisiae* fraction was extracted from cells growing exponentially, and not when it was extracted from cells growing in a stationary phase. Therefore, all proteases that are expressed constitutively, such as the vacuolar proteases, can be ruled out as the activating protease. Moreover, the timing of expression of this protease hints to the relevance *in vivo* of proteolytic activation of Chs2. The protease expression is during the exponential phase, when cells are dividing, which is consistent with the timing of Chs2 activity: the primary septum is synthesized after mitosis has completed [36]. Thus, this protease is expressed during the phase when Chs2 activity would peak.

Interestingly, a very similar type of proteolytic regulation is observed for chitin synthase from *M. sexta*, where the trypsin-activated serine protease CTLP1 was identified to interact with the chitin synthase [37]. Despite this similarity, the location and function of this insect chitin synthase are completely different to those of Chs2: the enzyme creates a protective matrix that lines the midgut of the larvae [38]. Similar modes of regulation for two chitin synthases from very different species, performing very different functions, suggest that this activation mechanism might be widespread.

Phosphorylation

Another fast and reversible way of enzyme regulation by post-translational modification is protein phosphorylation. To find out whether phosphorylation influences the activity of Chs2, we submitted *P. pastoris* membranes containing either Chs2 or Chs2ΔN-222 to dephosphorylation by the unspecific calf intestine alkaline

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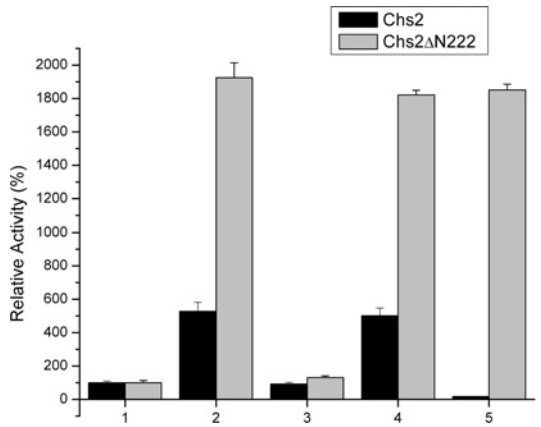

Figure 6 Phosphatase treatment of *P. pastoris*-expressed Chs2 (black) and Chs2ΔN222 (grey)

Activities are shown relative to their corresponding: (1) basal activities (non-treated membranes, 6.0 and 5.5 nmol of GlcNAc incorporated for Chs2 and Chs2ΔN222 respectively, each set to 100 %); (2) activities of trypsin-treated membranes; (3) activities of membranes treated with alkaline phosphatase; (4) activities of membranes treated with trypsin, followed by alkaline phosphatase; (5) activities of membranes treated with alkaline phoshphatase, followed by trypsin. Each bar comprises two independent sets of three experiments ± S.D. ($n = 6$).

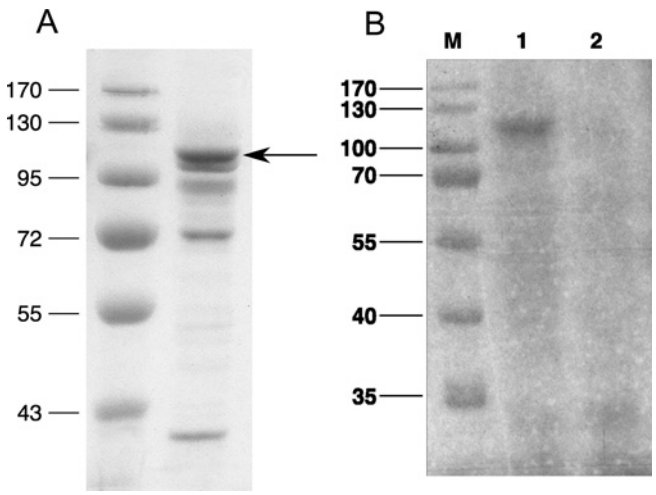

Figure 7 Phosphorylation analysis

(**A**) SDS/PAGE analysis of partially purified Chs2. Left lane: marker in kDa, right lane: Chs2 (115 kDa) partially purified by IMAC. The single purification step followed by SDS/PAGE allowed for the isolation of pure Chs2 for LC-MS/MS phosphorylation site mapping. (**B**) Autoradiography analysis of an SDS/PAGE gel from *P. pastoris* membranes containing overexpressed Chs2 or Chs2ΔN222 after treatment with Cdc2-cyclin B plus [γ-^{32}P]ATP. Per lane: M) marker in kDa, 1) membranes containing overexpressed Chs2 show a band at the molecular mass of the recombinant protein, 2) membranes containing overexpressed Chs2ΔN222 do not show a signal at the molecular mass of the recombinant protein.

Table 1 LC-MS/MS identification of phosphorylation sites on Chs2

The Table lists the phosphopeptides that were identified based on neutral loss of a mass of 98 and three continuous ion series. The residues denoted with an asterisk (*) are phosphorylated residues identified without ambiguity. The underlined residues are sites identified with neutral loss of a mass of 98 and only two continuous ion series. Sequence coverage was 56 %.

Starting amino acid	Phosphorylation sequence
4	NPFMVEPS*NGSPNR 17
4	NPFMVEPSNGS*PNR 17
36	WANPSEESLEDSYDQSNVFQGLPAS*PSR 63
64	AALRYSPDRR 73
93	YAANLQES*PKR 103
121	DNADLPVDPY*HLSPQQQPSNNLFGSGR 147
121	DNADLPVDPYHLS*PQQQPSNNLFGSGR 147
121	DNADLPVDPYHLSPQQQPSNNLFGS*GR 147
155	YTMSTTSTTAPSLAEADDEKEK 176
177	YLTS*TTSYDDQSTIFSADTFNETK 200
177	YLTSTTSYDDQSTIFS*ADTFNETK 200
254	RNSPEFTEMR 263

phosphatase. Dephosphorylation of membranes containing Chs2, whether trypsin-treated or not, does not significantly affect chitin synthase activity (Figure 6, conditions 3 and 4). Therefore, Chs2 does not appear to contain a phosphorylation site that plays a direct role in catalysis. However, when phosphatase treatment is followed by trypsinization, activity is almost completely lost (Figure 6, condition 5), indicating degradation of the protein. These results show that active Chs2 does contain one or more phosphorylation sites. These might, as for glycogen phosphorylase [39], be involved in organization and stabilization of the subunit structure of the enzyme. Similar experiments with Chs2ΔN222 show no effect after phosphatase treatment (Figure 6, condition 5), indicating that the phosphate groups that affect protein stability interact with or are located in the N-terminal domain.

Previous knowledge about phosphorylation of Chs2 is summarized in Figure 1. So far, Ser^{82} and Ser^{86} are the only phosphorylation sites that have been identified for wild-type Chs2 [40]. Additionally, Chs2 has been shown to be an *in vitro* substrate of the cyclin-dependent kinase Cdk1 [29,30]. To map the phosphorylation sites of Chs2, we analysed the purified enzyme by MS [41,42]. For this type of analysis, microgram amounts of pure protein are required. The level of Chs2 expression in *P. pastoris* allowed for purification of sufficient amounts of protein. We were able to solubilize the overexpressed enzyme in Fos-choline-14 detergent, and established an IMAC purification protocol using the His_{10} affinity tag. This single purification step significantly enriched the Chs2 content in the protein mixture, yielding ~50 % pure Chs2. The enzyme was further separated by SDS/PAGE (Figure 7A) and could be enriched to 100 % purity for mapping of phosphorylation sites by MS.

LC-MS/MS analysis identified 12 phosphorylation sites (Table 1), all in the N-terminal domain. The localization of the sites is consistent with our dephosphorylation experiments. Whereas Chs2 is affected by dephosphorylation, Chs2ΔN222, having only one of the 12 sites present, is not. Consequently, we can conclude that dephosphorylation of the N-terminal domain sensitizes the enzyme towards trypsin. Trypsin is a highly unspecific protease, which cleaves a polypeptide chain after almost every exposed positively charged amino acid residue. An elevated sensitivity towards trypsin usually indicates a less compactly folded and thus less stable protein. Dephosphorylation of the N-terminal domain clearly exposes and destabilizes parts of Chs2 that are stable when this domain is phosphorylated or not present at all. This hints at a regulatory function for (de)phosphorylation of the N-terminal domain, as for example an involvement in the short lifetime of the enzyme.

Expression, localization and degradation of Chs2 are cell-cycle dependent [15]. The enzyme is mainly expressed during mitosis [13]. It is transported from the endoplasmic reticulum to the mother–bud neck at mitotic exit, to synthesize the primary septum [27]. Finally, after cytokinesis it is proteolytically processed in the vacuole [14]. Such a complex cycle requires tight regulation of activity and stability. If phosphorylation is important for protein stability, the enzyme should be phosphorylated immediately after

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synthesis, and dephosphorylation could then facilitate rapid degradation of the protein when this becomes necessary.

In this light, it is interesting to note that four phosphorylation sites match the perfect phosphorylation motif (S/T-P-x-K/R) for Cdk1, a cyclin-dependent kinase that controls many cell cycle events, and that they are all located in the N-terminal domain (Figure 1). Furthermore, all four sites are observed in our LC-MS/MS experiments. Indeed, Chs2 has been revealed as an *in vitro* substrate of Cdk1, with the highest phosphorylation rate when it is complexed with Clb2 [29,30]. We have used the homologous human complex in combination with [γ-^{32}P]ATP and have shown that Chs2ΔN222 is not phosphorylated by this complex, whereas Chs2 is (Figure 7B). Thus, phosphorylation by Cdk1 is at the N-terminal domain, which is consistent with the phosphorylation sites observed with LC-MS/MS.

The N-terminal domains of chitin synthases can be quite different, and their regulatory role is uncertain [16]. However, CaChs1, the functional homologue of Chs2 from *C. albicans*, has an N-terminal domain that is homologous to that of Chs2, and contains three perfect Cdk1 phosphorylation motifs. Indeed, all N-terminal domains that are homologous to that of Chs2 (UniProtKB primary accession numbers Q6FQN5, A7TEI4, Q6CS65, Q758F2 and Q6BZ46) contain multiple perfect Cdk1 phosphorylation sites, in most cases even in the same position as for Chs2. Therefore, it appears that the Cdk1 phosphorylation sites are a feature of this family of N-terminal domains, strengthening the suggestion of a functional role.

Chs2 is most efficiently phosphorylated when Cdk1 is complexed with Clb2 [30]. As this is an M-phase cyclin, it is active during mitosis, when Chs2 is synthesized, but not after cytokinesis, when Chs2 is degraded. Cdk1–Clb2 phosphorylates the N-terminal domain of Chs2, and the timing of its activity is correlated with that of Chs2. As tempting as it is to speculate about a possible role for Cdk1 in regulating Chs2 activity, site-directed mutagenesis studies will be necessary to confirm whether these specific phosphorylation sites are of importance for the short lifetime of Chs2.

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REFERENCES

1 Cabib, E., Roberts, R. and Bowers, B. (1982) Synthesis of the yeast cell wall and its regulation. Ann. Rev. Biochem. **51**, 763–793
2 Shaw, J. A., Mol, P. C., Bowers, B., Silverman, S. J., Valdivieso, M. H., Durán, A. and Cabib, E. (1991) The function of chitin synthases 2 and 3 in the *Saccharomyces cerevisiae* cell cycle. J. Cell Biol. **114**, 111–123
3 Silverman, S. J. (1989) Similar and different domains of chitin synthases 1 and 2 of *S. cerevisiae*: two isozymes with distinct functions. Yeast **5**, 459–467
4 Campbell, J. A., Davies, G. J., Bulone, V. and Henrissat, B. (1997) A classification of nucleotide-diphospho-sugar glycosyltransferases based on amino acid sequence similarities. Biochem. J. **326**, 929–939
5 Coutinho, P. M., Deleury, E., Davies, G. J. and Henrissat, B. (2003) An evolving hierarchical family classification for glycosyltransferases. J. Mol. Biol. **328**, 307–317
6 Charnock, S. J. and Davies, G. J. (1999) Structure of the nucleotide-diphospho-sugar transferase, SpsA from *Bacillus subtilis*, in native and nucleotide-complexed forms. Biochemistry **38**, 6380–6385
7 Cabib, E., Sburlati, A., Bowers, B. and Silverman, S. J. (1989) Chitin Synthase 1, an auxiliary enzyme for chitin synthesis in *Saccharomyces cerevisiae*. J. Cell Biol. **108**, 1665–1672
8 Silverman, S. J., Sburlati, A., Slater, M. L. and Cabib, E. (1988) Chitin synthase 2 is essential for septum formation and cell division in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. U.S.A. **85**, 4735–4739
9 Bulawa, C. E., Slater, M., Cabib, E., Au-Young, J., Sburlati, A., Adair, W. L. and Robbins, P. W. (1986) The *S. cerevisiae* structural gene for chitin synthase is not required for chitin synthesis *in vivo*. Cell **46**, 213–225
10 Valdivieso, M. H., Mol, P. C., Shaw, J. A., Cabib, E. and Durán, A. (1991) *CAL1*, a gene required for activity of chitin synthase 3 in *Saccharomyces cerevisiae*. J. Cell Biol. **114**, 101–109
11 Nagahashi, S., Sudoh, M., Ono, N., Sawada, R., Yamaguchi, E., Uchida, Y., Mio, T., Takagi, M., Arisawa, M. and Yamada-Okabe, H. (1995) Characterization of chitin synthase 2 of *Saccharomyces cerevisiae*. Implication of two highly conserved domains as possible catalytic sites. J. Biol. Chem. **270**, 13961–13967
12 Munro, C. A., Winter, K., Buchan, A., Henry, K., Becker, J. M., Brown, A. J. P., Bulawa, C. E. and Gow, N. A. R. (2001) Chs1 of *Candida albicans* is an essential chitin synthase required for synthesis of the septum and for cell integrity. Mol. Microbiol. **39**, 1414–1426
13 Choi, W.-J., Santos, B., Durán, A. and Cabib, E. (1994) Are yeast chitin synthases regulated at the transcriptional or the posttranslational level? Mol. Cell. Biol. **14**, 7685–7694
14 Chuang, J. S. and Schekman, R. W. (1996) Differential trafficking and timed localization of two chitin synthase proteins, Chs2p and Chs3p. J. Cell Biol. **135**, 597–610
15 Roncero, C. (2002) The genetic complexity of chitin synthesis in fungi. Curr. Genet. **41**, 367–378
16 Ford, R. A., Shaw, J. A. and Cabib, E. (1996) Yeast chitin synthases 1 and 2 consist of a non-homologous and dispensable N-terminal region and of a homologous moiety essential for function. Mol. Gen. Genet. **252**, 420–428
17 André, N., Cherouati, N., Prual, C., Steffan, T., Zeder-Lutz, G., Magnin, T., Pattus, F., Michel, H., Wagner, R. and Reinhart, C. (2006) Enhancing functional production of G protein-coupled receptors in *Pichia pastoris* to levels required for structural studies via a single expression screen. Prot. Science **15**, 1115–1126
18 Sburlati, A. and Cabib, E. (1986) Chitin synthetase 2, a presumptive participant in septum formation in *Saccharomyces cerevisiae*. J. Biol. Chem. **261**, 15147–15152
19 Au-Young, J. and Robbins, P. W. (1990) Isolation of a chitin synthase gene (*CHS1*) from *Candida albicans* by expression in *Saccharomyces cerevisiae*. Mol. Microbiol. **4**, 197–207
20 Fähnrich, M. and Ahlers, J. (1981) Improved assay and mechanism of the reaction catalyzed by the chitin synthase from *Saccharomyces cerevisiae*. Eur. J. Biochem. **121**, 113–118
21 Sudoh, M., Yamazaki, T., Masubuchi, K., Taniguchi, M., Shimma, N., Arisawa, M. and Yamada-Okabe, H. (2000) Identification of a novel inhibitor specific to the fungal chitin synthase - inhibition of chitin synthase 1 arrests the cell growth, but inhibition of chitin synthase 1 and 2 is lethal in the pathogenic fungus *Candida albicans*. J. Biol. Chem. **275**, 32901–32905
22 Uchida, Y., Shimmi, O., Sudoh, M., Arisawa, M. and Yamada-Okabe, H. (1996) Characterization of chitin synthase 2 of *Saccharomyces cerevisiae* II: both full size and processed enzymes are active for chitin synthesis. J. Biochem. **119**, 659–666
23 Persson, K., Ly, H. D., Dieckelmann, M., Wakarchuk, W. W., Withers, S. G. and Strynadka, N. C. J. (2001) Crystal structure of the retaining galactosyltransferase LgtC from *Neisseria meningitidis* in complex with donor and acceptor sugar analogs. Nat. Struct. Biol. **8**, 166–175
24 Choi, W.-J. and Cabib, E. (1994) The use of divalent cations and pH for the determination of specific yeast chitin synthetases. Anal. Biochem. **219**, 368–372
25 Schmidt, M., Bowers, B., Varma, A., Roh, D.-H. and Cabib, E. (2002) In budding yeast, contraction of the actomyosin ring and formation of the primary septum at cytokinesis depend on each other. J. Cell Sci. **115**, 293–302
26 Tolliday, N., Pitcher, M. and Li, R. (2003) Direct evidence for a critical role of myosin II in budding yeast cytokinesis and the evolvability of new cytokinetic mechanisms in the absence of myosin II. Mol. Biol. Cell **14**, 798–809
27 Zhang, G., Kashimshetty, R., Ng, K. E., Tan, H. B. and Yeong, F. M. (2006) Exit from mitosis triggers Chs2p transport from the endoplasmic reticulum to mother-daughter neck via the secretory pathway in budding yeast. J. Cell Biol. **174**, 207–220
28 Roh, D.-H., Bowers, B., Schmidt, M. and Cabib, E. (2002) The septation apparatus, an autonomous system in budding yeast. Mol. Biol. Cell **13**, 2747–2759
29 Ubersax, J. A., Woodbury, E. L., Quang, P. N., Paraz, M., Blethrow, J. D., Shah, K., Shokat, K. M. and Morgan, D. O. (2003) Targets of the cyclin-dependent kinase Cdk1. Nature **425**, 859–864
30 Loog, M. and Morgan, D. O. (2005) Cyclin specificity in the phosphorylation of cyclin-dependent kinase substrates. Nature **434**, 104–108

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31 Cabib, E. and Farkas, V. (1971) The control of morphogenesis: enzymatic mechanism for initiation of septum formation in yeast. Proc. Natl. Acad. Sci. U.S.A. **68**, 2052–2056
32 Zimoch, L., Hogenkamp, D. G., Kramer, K. J., Muthukrishnan, S. and Merzendorfer, H. (2005) Regulation of chitin synthesis in the larval midgut of *Manduca sexta*. Insect Biochem. Mol. Biol. **35**, 515–527
33 Ulane, R. E. and Cabib, E. (1976) The activating system of chitin synthetase from *Saccharomyces cerevisiae* - purification and properties of activating factor. J. Biol. Chem. **251**, 3367–3374
34 Zubenko, G. S., Mitchell, A. P. and Jones, E. W. (1979) Septum formation, cell division, and sporulation in mutants of yeast deficient in proteinase B. Proc. Natl. Acad. Sci. U.S.A. **76**, 2395–2399
35 Silverman, S. J., Shaw, J. A. and Cabib, E. (1991) Proteinase B is, indeed, not required for chitin synthetase 1 function in *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. **174**, 204–210
36 Cabib, E. (2004) The septation apparatus, a chitin-requiring machine in budding yeast. Arch. Biochem. Biophys. **426**, 201–207
37 Broehan, G., Zimoch, L., Wessels, A., Ertas, B. and Merzendorfer, H. (2007) A chymotrypsin-like serine protease interacts with the chitin synthase from the midgut of the tobacco hornworm. J. Exp. Biol. **210**, 3636–3643
38 Merzendorfer, H. (2006) Insect chitin synthases: a review. J. Comp. Physiol. B **176**, 1–15
39 Johnson, L. N. (1992) Glycogen phosphorylase: control by phosphorylation and allosteric effectors. FASEB J. **6**, 2274–2282
40 Ficarro, S. B., McCleland, M. L., Stukenberg, P. T., Burke, D. J., Ross, M. M., Shabanowitz, J., Hunt, D. F. and White, F. M. (2002) Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*. Nat. Biotechnol. **20**, 301–305
41 Gafken, P. R. and Lampe, P. D. (2006) Methodologies for characterizing phosphoproteins by mass spectrometry. Cell Commun. Adhes. **13**, 249–262
42 Zheng, X.-D., Lee, R. T. H., Wang, Y.-M., Lin, Q. S. and Wang, Y. (2007) Phosphorylation of Rga2, a Cdc42 GAP, by CDK/Hgc1 is crucial for *Candida albicans* hyphal growth. EMBO J. **26**, 3760–3769

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